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Plasmolipin: genomic structure, chromosomal localization, protein expression pattern, and putative association with Bardet-Biedl syndrome

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Abstract. Plasmolipin is a membrane protein and belongs to the tetraspan molecule (4TM) family, an expanding group of myelin proteins many of which could be linked to human hereditary demyelinating neuropathies. We have cloned and sequenced the mouse plasmolipin gene, revealing the common organization of the 4TM gene group with four exons and a large first intron. Western blot analysis with an antibody raised against the C-terminal intracellular part of the protein showed that plasmolipin is expressed not only in the nervous system and kidney, but also in a number of other tissues such as thymus, testis, lung, and thyroid gland. By means of radiation hybrid mapping and FISH analysis, we could localize the human plasmolipin gene to Chromosome 16q13 within the putative region of the Bardet-Biedl syndrome type 2 (BBS2) gene locus. BBS2 is a clinically and genetically heterogeneous group of disorders resulting in rod-cone dystrophy, obesity, postaxial polydactyly, renal dysfunction, and mental retardation, which were very recently associated with a novel gene designated BBS2. With respect to intrafamiliar variations in the manifestation of BBS, we suggest that plasmolipin might be either another candidate gene or a modifier of the BBS2 phenotype.

Plasmolipin was initially isolated from kidney plasma membranes as a protein of 20 kDa (Tosteson and Sapirstein 1981) and classified as a proteolipid (Lees et al. 1979) owing to its high proportion of hydrophobic amino acids and its solubility in organic solvents.

Subsequent Northern blot analysis detected plasmolipin expression in Schwann cells of sciatic nerves and oligodendrocytes in brain (Cochary et al. 1990; Fischer et al. 1991). Developmental analysis indicated a strong temporal correlation between plasmolipin mRNA-expression and postnatal myelination in the peripheral and central nervous system, as well as remyelination during sciatic nerve regeneration (Gillen et al. 1996). Furthermore, topographic models based on the nucleotide sequence predict four putative transmembrane regions (Gillen et al. 1996), a cardinal feature of the tetraspan molecule family, an expanding group of myelin proteins including proteolipid protein (PLP; Milner et al. 1985), peripheral myelin protein 22 kDa (PMP22; Spreyer et al. 1991) and myelin and lymphocyte protein (MAL; Schaeren-Wiemers et al. 1995). Most of them have been shown to be involved in causing hereditary demyelinating neuropathies like PMP22 in Charcot-Marie-Tooth 1A (for review see Müller 2000) and PLP in Pelizaeus-Merzbacher disease (Hudson et al. 1989). Unfortunately, little is known about the biological function of plasmolipin in vivo and its potential relation to neurological diseases.

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In the present investigation, we have determined the genomic structure, protein expression pattern, and chromosomal localization of plasmolipin. Surprisingly, we detected the plasmolipin gene near the chromosomal locus of the Bardet-Biedl syndrome (BBS, MIM 209900). BBS is a generic description for a clinically and genetically heterogeneous group of disorders resulting in rod-cone dystrophy, obesity, postaxial polydactyly, hypogenitalism, renal dysfunction, and mental retardation (Bardet 1995; Biedl 1995; Green et al. 1989; Harnett et al. 1988). Because of this widespread spectrum of features overlapping with those of the Laurence-Moon syndrome (additional spastic paraplegia, rare polydactyly) a new description label, polydactyly-obesity-kidney-eye syndrome, was proposed recently (Beales et al. 1999).

Interestingly, not every BBS patient shows all known symptoms, and within affected families the symptoms may vary (Green et al. 1989; Beales et al. 2000). Nearly all suffer from rod-cone dystrophy (approx. 90%), but only approx. 70% have postaxial polydactyly, and fewer reveal developmental delay (approx. 50%) or ataxia (approx. 40%; Beales et al. 1999). BBS is linked to at least six chromosomal loci: BBS1 to Chromosome (Chr) 11q13 (Leppert et al. 1994); BBS2 to Chr 16q13–21 with the gene order cen–D16S408–2cM–BBS2–5cM–D16S400 (Kwitek-Black et al. 1993); BBS3 to Chr 3p13 (Sheffield et al. 1994); BBS4 to Chr 15q23 (Carmi et al. 1995); BBS5 to Chr 2q31 (Young et al. 1999); and BBS6 to Chr 20p12 (Katsanis et al. 2000; Slavotinek et al. 2000).

Thus far, BBS6 could be linked to a distinct gene, namely the McKusick-Kaufman syndrome gene (MKKS gene; Katsanis et al. 2000; Stone et al. 2000; Slavotinek et al. 2000), while most other candidate genes for other BBS types could not be verified up to now. In general, some authors suggest chaperonins may be involved in causing this syndrome (Slavotinek et al. 2000), molecular pathways that could be influenced by the genomic background (Riise et al. 1997) or epistatic interactions between BBS genes leading to abnormal precursor cell growth or altered differentiation (Beales et al. 2000).

Materials and methods

Screening of a genomic mouse library. The genomic mouse 129/SvevTACfBr P1 library RPCI 21 (414750 clones, average insert size 146.6 kb), constructed at the Roswell Park Cancer Institute by Osoegawa and de Jong, was provided by the Ressourcen Zentrum/Primär Datenbank (RZPD) of the German Human Genome Project. Filters were prehybridized in Church-buffer (7% SDS, 0.5 M sodium phosphate pH 7.2) at 68°C for 1 h. A rat plasmolipin cDNA spanning from nucleotides 372 to 1476 was labeled with [α^{32}P]dCTP by using Ready-To-Go DNA Labeling Beads (Pharmacia). Hybridization was carried out in Church-buffer at 68°C overnight. Filters were washed twice in 0.1% SDS, 40 mM sodium phosphate (pH 7.2) at 25°C for 5 min, and two times at 68°C for 30 min. Autora-

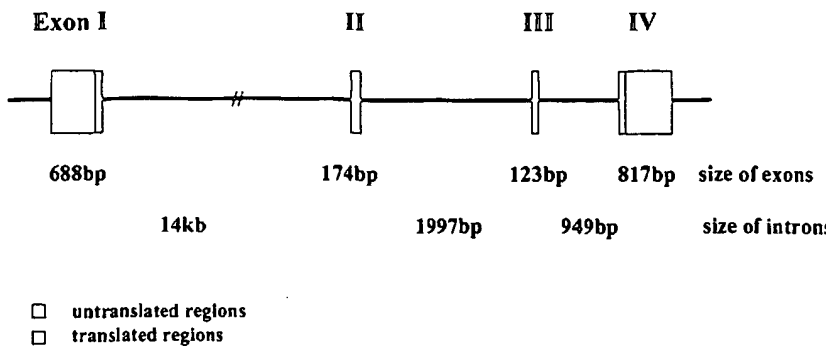


Fig. 1. Genomic structure of the mouse plasmolipin gene. The plasmolipin gene spans approx. 19 kb and consists of four exons. Exons are indicated as squares, coding regions are marked in black, untranslated regions remain unfilled.

diographic signals detected by exposure to X-ray films (Kodak) were assigned to the filter coordinates of the corresponding P1 clones.

Subcloning and sequencing. The PAC clone RPCIP711E01102Q2 was cut with the restriction enzymes *Bam*HI and *Eco*RI, respectively, and subcloned into pBluescript KS II (Invitrogen). Colonies were transferred onto C/P lift membranes (Biorad), denatured with 1.5 M NaCl, 0.5 M NaOH, and neutralized with 1.5 M NaCl, 1 M Tris-HCl (pH 7.0). Hybridizations with a full-length rat plasmolipin cDNA probe (nucleotides 216–1425), a 5′ probe (nucleotides 216–802), and a 3′ probe (nucleotides 769–1425), respectively, were performed as described above. Sequencing of positive clones was carried out by means of primer walking with an ABI 310 genetic analyzer (PE Biosystems). Sequence data analysis was performed with Sequencher 4.0.5 (Gene Codes) and BLASTN (Genome project Japan) softwares.

A 2-kb part of the genomic gene sequence missing between the first and second exon was generated by PCR, by using *Pfu* polymerase and two primers that were designed from the sequence of two screened clones (primers Pi41 5′-TAGAGGAATAGGGTAGCACCGCAG-3′, Mi33 5′-GTCCCTGGCCTAGATCTCC-3′). PCR conditions were: 95°C for 2 min; 94°C for 30 s, 54°C for 45 s, and 72°C for 2 min (25 cycles), followed by a 10-min extension at 72°C in a standard mix. PCR products were subcloned into pCR vector with the Zero Blunt PCR Cloning Kit (Invitrogen). The sequence of the mouse plasmolipin gene is available under the following Genbank accession numbers: AJ298129 and AJ298130.

Genomic mapping.

a) Radiation hybrid mapping: The Stanford G3 Radiation Hybrid Panel was used to determine the genomic localization of the human plasmolipin gene. This panel consists of 83 hybrid cell lines derived from fusion between a hamster cell line and γ-irradiated human cells. Two different sets of human plasmolipin-specific oligonucleotides were tested in Hot-Star-PCR (Qiagen): (i) hPla-P1: 5′-GCGCCTGGAGCCACACAG-3′; hPla-M1: 5′-GCCATGGCGGCTCCGCTTGC-3′ and (ii) hPla-P2: 5′-CTGGCAGGCATCTCGGCTTGC-3′; hPla-M2: 5′-GAGAGTGGTTTCACCAGCTTGC-3′. PCR reactions were set up in a volume of 50 µl containing 12.5 ng genomic DNA of each of the hybrid cell lines, respectively. The samples were cycled 34 times for 45 s at 94°C, 30 s at 62°C, and 45 s at 72°C, and separated on 1.5% agarose gels. Results of positive hybrid cell lines were translated into a binary code and analyzed by the Radiation Hybrid Map Search Engine (version 2.0; Stanford Human Genome Center).

b) Fluorescence in situ hybridization: FISH analysis was carried out on normal metaphase chromosomes from cultured peripheral human blood. Appropriate GTG-banded metaphases were photographed, and slides were destained. The cosmid probe pWE-1 from a human placenta genomic library (Stratagene) containing a human plasmolipin gene fragment (data not shown) was labeled with digoxigenin-11-dUTP by standard nick translation (Gibco BRL). Hybridization was performed overnight at 37°C. Posthybridization washes were carried out in 50% formamide/2×SSC at 43°C and 2×SSC at 37°C, followed by detection with fluorescein-labeled anti-digoxigenin antibodies and amplification of the signal (detection kit, Oncor Appligene). Slides were counterstained with propidium iodide and visualized under a Zeiss Axiophot fluorescence microscope. Relocated metaphases were photographed with Kodak Ektachrome 400 film.

Protein preparation. Tissues of neonatal Wistar rats were shock frozen in liquid nitrogen and homogenized in a dounce homogenizer in 10 mM HEPES-buffer pH 7.4 containing 2 mM $MgCl_2$, 2 mM pefabloc, 1 mM leupeptin, and 1 mM pepstatin (all Roche). The homogenates were centrifuged at 4°C and 10,000 *g* for 15 min, and the supernatants were then centrifuged at 4°C and 100,000 *g* for 1 h. The pellet containing peripheral and integral membrane proteins was solubilized overnight in PBS with 2% CHAPS (Roth Chemicals). The CHAPS concentration was brought to 0.5% by adding PBS, and the solution was cleared by centrifugation for 15 min (40,000 *g*).

Production of plasmolipin antibodies, SDS-PAGE, and Western blotting. Synthesis of peptides and immunization of rabbits were carried out by Antibody Service Dr. Pineda (Berlin), by using the peptide plapi4 corresponding to amino acids 166–182 (RGVGSNAATSQMAGGYS) of rat plasmolipin. SDS–PAGE was performed on 12% acrylamide-gels under non-reducing conditions. Gels were stained with Coomassie Blue (Serva) to verify equal amounts of total protein. For Western blotting, 30 µg of protein was separated electrophoretically and transferred to nitrocellulose membranes in a semidry blotting chamber (Biorad). Free binding sites were blocked overnight at 4°C with 3% nonfat dry milk powder/1% BSA in Tris-buffered saline containing 0.05% Tween 20 (TBS-T). Antibody incubation was performed for 1 h at room temperature in TBS-T. Detection of immune complexes was carried out by incubation with peroxidase-coupled goat anti-rabbit immunoglobulins (Southern Biotechnology Association) and the ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

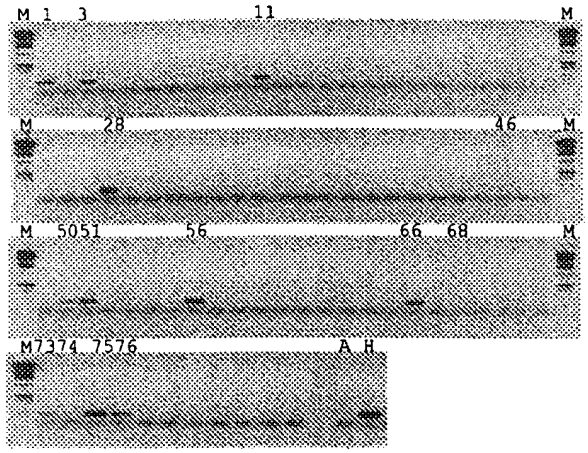
Results

Genomic structure of the mouse plasmolipin gene. Screening of the genomic mouse P1 library No. 711 with a plasmolipin cDNA full-length probe revealed 13 positive clones, corresponding well with the library feature to present 12.6 times the mouse genome. As all clones showed similar restriction patterns (data not shown), one clone, RPCIP711E01102, was subcloned, and the resulting clones were rescreened. Five partially overlapping subclones were identified and sequenced by means of primer walking. A 2-kb fragment missing within the genomic gene sequence of the subclones was generated from genomic mouse DNA with the help of PCR, by using *Pfu* polymerase, and sequenced.

As plasmolipin is phylogenetically a new gene that is restricted to mammals (Sapirstein et al. 1991) and presumably conserved

Table 1. Exon-intron boundaries of the mouse plasmolipin gene.

Exon	Exon Size (bp)	Intron Size (bp)	Donor	Acceptor
I	688	~14000	CTG gtgagc	tttccacag GCAC
II	174	1997	GTG gtgagt	cccctacag TTAC
III	123	949	TCT gtaagt	ccctgccag TTCT
IV	817			



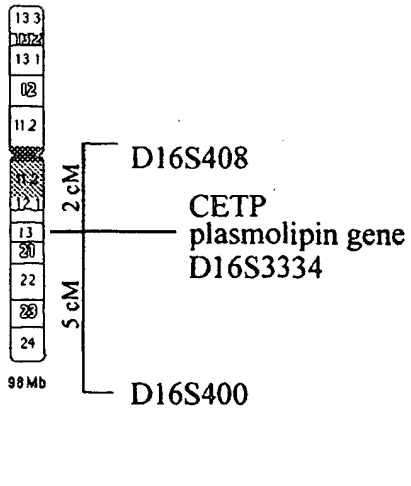


Fig. 2. Chromosomal localization of the human plasmolipin gene. **A.** Radiation hybrid mapping using the SHGC Panel G3 was carried out with primers hPla-M1 and hPla-P1 or hPla-M2 and hPLA-P2 according to the SGHC protocol and revealed the same amplification pattern. Hybrids with amplification products are indicated: M, 1-kb marker; A, hamster DNA (negative control); H, human DNA (positive control). **B.** Position of the plasmolipin gene on human Chr 16q13. The plasmolipin gene is located next to the SHGC markers D16S3334 and CETP, approx. 2 cM from marker D16S408 and approx. 5 cM from marker D16S400.

(homology between human and rat cDNAs, 86%), an alignment search against rat and human plasmolipin cDNAs (Gillen et al. 1996, EMBL Z49858; Xie et al. unpublished, EMBL AF137386) was performed. Computer-assisted contig analysis revealed that the mouse plasmolipin gene is approx. 19 kb in length and consists of four exons (Fig. 1; EMBL AJ298129 and AJ298130). Exon-intron boundaries were determined via SpliceView of the Institute of Advanced Biomedical Technologies (ITBA, Italy; http://125.itba.mi.cnr.it/~webgene/wwwspliceview.html) and revealed to be consistent with the GT/AG rule (Table 1). The start codon has been found in exon I, while the polyadenylation signal is within exon IV. Comparison with rat cDNA revealed, indeed, a high homology of 93.7% within the coding region. Most nucleotide substitutions are silent mutations or result in an exchange of a hydrophobic amino acid with another. A transversion A634C (corresponding to the rat cDNA) leads to a leucine instead of a methionine within the first putative intracellular loop.

Chromosomal localization of the human plasmolipin gene. In order to determine the chromosomal localization of the human plasmolipin gene, a G3 radiation hybrid panel system of Stanford University was used. PCR with two different pairs of human plasmolipin cDNA specific primers revealed identical amplification patterns, as shown in Fig. 2A. Results were transformed into a binary code and analyzed at the Stanford Human Genome Center RHserver. The human plasmolipin gene was localized on Chr 16q13 near the marker SHGC-34581 with a LOD-score of 11.48 between CETP and D16S3334 (Fig. 2B). An independent FISH analysis also confirmed this chromosomal localization. Metaphase chromosomes were identified on the basis of size, morphology, and banding pattern (Fig. 3A, C). Hybridization with a digoxigenin-labeled human plasmolipin gene fragment showed positive signals on Chr 16q13 (Fig. 3B, D). The critical region for the Bardet-Biedl syndrome type 2 (BBS2; Kwitek-Black et al. 1993) is localized in this region with the proposed gene order cen–D16S408–2cM–BBS2 gene–5cM–D16S400.

Tissue distribution of rat plasmolipin protein expression. The antibody pla-4 directed against the C-terminal intracellular part of rat plasmolipin specifically recognized a 20-kDa band on Western blots of total protein isolated from rat sciatic nerve and brain (Fig. 4A). No staining was obtained when using preimmune serum (data not shown).

In addition, plasmolipin protein was detected in spinal cord (Fig. 4A), parts of the intestinal tract including stomach and esophagus (Fig. 4B), in kidney, heart muscle, lung, thymus, ovary, and testis (Fig. 4C), as well as in endocrine and exocrine glands, namely, suprarenal gland, parotic gland, submandibular gland, cowper gland, prostate gland, and thyroid gland (Fig. 4D). Plasmolipin protein was expressed at low levels in colon (Fig. 4B) and skeletal muscle (Fig. 4C), but was not detectable in retina (data not shown), jejenum and ileum (Fig. 4B), spleen (Fig. 4C), pancreas and liver (Fig. 4D). In several tissues, e.g., thymus and spinal cord, additional protein bands occurred, which possibly result from multimerization or degradation, respectively (data not shown).

Discussion

We have isolated the mouse plasmolipin gene and have determined its genomic structure. Plasmolipin consists of four exons and a large intron I. The nucleotide sequence of plasmolipin cDNA predicts a topographic model with four putative transmembrane domains (TM; Fischer and Sapirstein 1994; Gillen et al. 1996). This model is supported by the fact that each TM is encoded by a single exon in mouse, except TM I, which is encoded half by exon I and exon II (data not shown). A comparison with the genomic structure of the myelin proteins PMP22 (for review see Taylor et al. 2000) and MAL (Magyar et al. 1997) reveals a similar gene organization, indicating a common feature of this subfamily of tetraspan molecules. In general, the coding region of plasmolipin is highly conserved throughout the mammalian species rat, mouse, and human as shown here, with an average homology of approximately 90%.

Western blot analysis revealed that rat plasmolipin protein is present in a number of tissues including kidney and the nervous system, consistent with results obtained with Northern blot analysis described elsewhere (Gillen et al. 1996). Furthermore, we could detect plasmolipin protein expression in spinal cord, in intestinal tract, in ovary, in testis, as well as in suprarenal and thyroid glands. On the other hand, plasmolipin protein was not found in retina, spleen, and liver. In general, all tissues positive for plasmolipin are specialized organs with a high degree of complexity, containing polarized cells or cell layers. It was shown recently that the 4TM

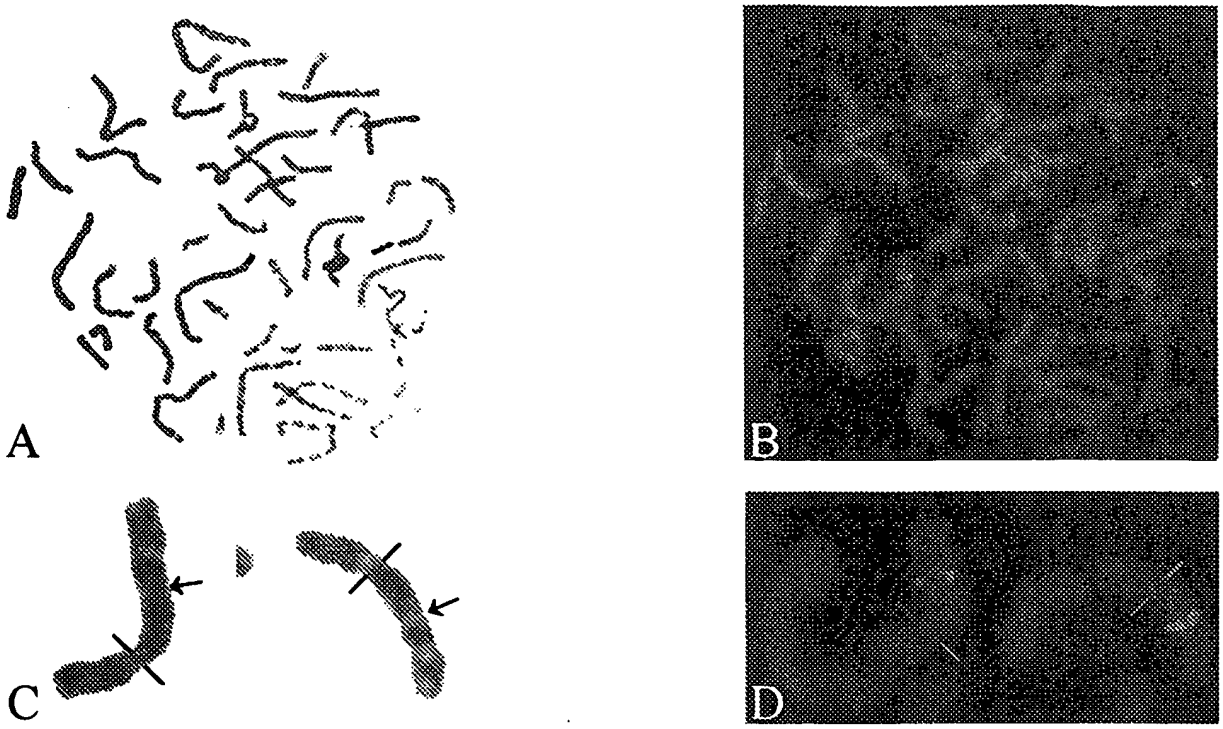


Fig. 3. Localization of plasmolipin by FISH. After GTG-banding of the metaphase (**A, C**), the same metaphase was hybridized with the cosmid pWE-1 (**B, D**) containing a part of the human plasmolipin gene. Amplified fluorescein-signals on propidium iodide counterstained chromosomes are clearly visible on Chr 16q13.

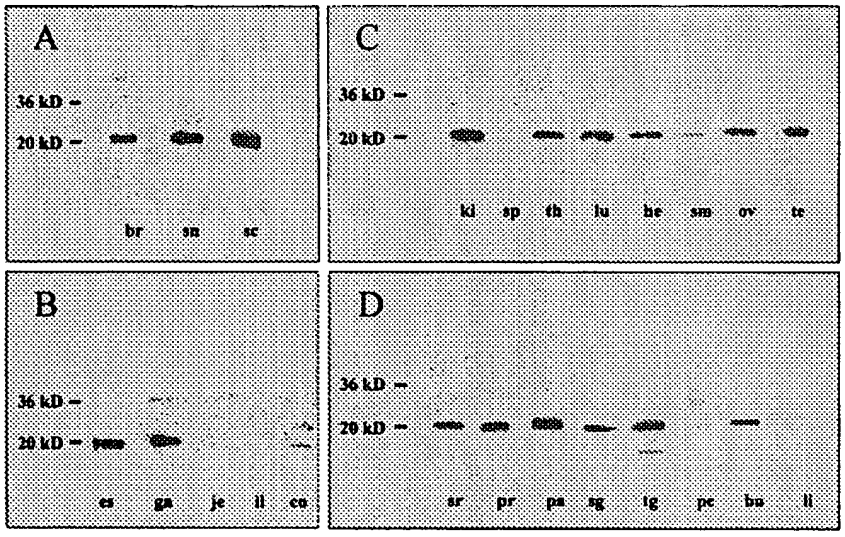


Fig. 4. Detection of plasmolipin in membrane fractions of different rat tissues. 30 μg of total protein was separated on a 15% SDS-PAGE, then probed with the plasmolipin polyclonal antibody pla-4. **A.** Protein expression in the rat nervous system; br (brain), sn (sciatic nerve), sc (spinal cord). **B.** Intestinal tract; es (esophagus), ga (stomach), je (jejunum), il (ileum), co (colon). **C, D.** Further tissues: ki (kidney), sp (spleen), th (thymus), lu (lung), he (heart), sm (skeletal muscle), ov (ovar), te (testis), sr (suprarenal gland), pr (prostate gland), pa (parotic gland), sg (submandibular gland), tg (thyroid gland), pc (pancreas), bu (cowper gland), and li (liver).

protein MAL is involved in the apical sorting machinery of polarized cells, e.g., in MAL overexpressing Sf21 insect cells (Puertollano et al. 1997) and MDCK cells (Martin-Belmonte et al. 2000). As both proteins, MAL and plasmolipin, share common topographical features, it seems possible that plasmolipin also could play a similar role in cellular protein targeting.

Since mutations in the genes of the tetraspan myelin proteins PMP22, Connexin 32, and PLP cause hereditary demyelinating diseases such as CMT1A (for review see Müller 2000), CMTX (Bergoffen et al. 1993) and Pelizaeus-Merzbacher disease (Hudson et al. 1989), respectively, it has been anticipated that the 4TM myelin protein plasmolipin may also relate to a hereditary neurological disorder. By means of two independent methods, we have localized the human plasmolipin gene on Chr 16q13 within the critical region for the Bardet-Biedl syndrome type 2 (BBS2; see Kwitek-Black et al. 1993).

Recently, a C-terminal kinesin (KIFC3), a member of the large superfamily of microtubule motors, has been proposed as a candidate gene for BBS2 (Hoang et al. 1998). However, homozygous KIFC3 knockout mouse mutants develop and reproduce normally (Yang et al. 2001), indicating that this kinesin motor may not contribute to BBS2 alone. Sheffield and collegues reported a novel gene also localized within the BBS2 gene locus (Nishimura et al. 2001). In some BBS2 patients, mutations were found in this gene, which was, therefore, proposed as a candidate gene and was designated BBS2. Unfortunately, no functional data of the BBS2 pro-

tein are available up to now. The remarkable heterogeneity of this syndrome even in kindreds suggests that more than one gene might be affected to explain the observed phenotypic variations.

The plasmolipin gene is localized well within the critical region for the BBS2 on Chr 16q13 close to the KIFC3 and BBS2 genes. In addition, plasmolipin is expressed in nearly all tissues that are affected in patients suffering from BBS2, including kidney and the peripheral nervous system. Therefore, the plasmolipin protein could interfere in molecular pathways in which the BBS2 protein is involved, e.g., in case plasmolipin might play a role in the protein trafficking machinery of the cell. Thus, the plasmolipin gene might be another candidate gene revealing mutations not in all BBS2 patients and/or showing altered expression levels in affected persons. It is also possible that plasmolipin is a modifier of the BBS2 phenotype and affects this syndrome depending on the genomic background or secondary features. Both ideas are supported by the fact that plasmolipin protein was not found in the retina, though rod-cone dystrophy is a cardinal feature of BBS. This leads to the suggestion that the plasmolipin gene might either contribute to retina degeneration via secondary effects or, on the other hand, could be affected in a number of BBS2 patients in addition to another gene or several other genes. Both mechanisms could explain the complex heterogeneity of the BBS2 syndrome.

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